U.S. Army Center for Health Promotion and Preventive Medicine

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TOXICOLOGY STUDY NO. 87-XE-0982-09
IN VITRO STUDY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) METABOLISM IN HUMAN LIVER

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14. ABSTRACT

This project is the first to investigate RDX metabolism in human liver. RDX metabolism was screened in human liver tissues including S9 preparations, microsomes, hepatocytes and several recombinant CYP450 isoforms under aerobic, anaerobic and oxygen reduced conditions. RDX metabolism was also conducted in several animal liver microsomes to compare with human liver microsomes. loss of the parent compound (RDX) was determined at 30 and 180 minutes of the incubation and ranked as human (46.6% & 51.8%)> rat (40.1% & 47.2%)> monkey (34.6% & 35.7%)> Pig (25.5% & 33.7%)> rabbit (11.6% & 18.0). The data is used to establish Physiologically-based pharmacokinetic (PBPK) models for human useful in risk assessment. Further characterization of the profiles of RDX in vitro metabolism in human and animal liver tissues is proposed. This study developed an in vitro metabolic model which mimics in vivo physiological condition and will be useful in the evaluation of human metabolic fate for novel energetics such as replacement formulations for RDX and for other toxic TICs/TIMs.

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U.S. Army Corps of Engineers Engineering Engineer, Research and Development Center
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IN VITRO STUDY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) METABOLISM IN HUMAN LIVER
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Performing Laboratory

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Organization: U.S. Army Corps of Engineers, Engineer, Research and Development Center (ERDC)

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EXECUTIVE SUMMARY
TOXICOLOGY STUDY NO. 87-XE-0982-09
IN VITRO STUDY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) METABOLISM IN HUMAN LIVER

1. PURPOSE. This project addresses RDX metabolism in human liver. These studies were based on the use of human tissues and cells to investigate RDX metabolism in human liver in support of the current re-assessment of RDX being carried out by the U.S. Environmental Protection Agency (USEPA) with supporting information from the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Directorate of Toxicology (DTOX). It is anticipated that a refined RfD (oral reference dose) for RDX will be developed by the USEPA. This project was conducted under the work unit of pharmokinetics and toxicodynamics of RDX within the Toxicogenomics for Assessment of Munitions Constituents Program to provide more precise information useful in estimating the exposure hence the risk of humans exposed to environmental concentrations of RDX.

2. CONCLUSIONS.

a. This project is the first to investigate RDX metabolism in human liver. RDX metabolism was screened in human liver tissues including S9 preparations, microsomes, hepatocytes and several recombinant CYP450 isoforms under aerobic, anaerobic and oxygen reduced conditions. The RDX metabolism was also conducted in several animal liver microsomes to compare with human liver microsomes. Metabolism rates of RDX were determined after 30 and 180 minutes of the incubations in these enzymatic systems and ranked (from highest to lowest) as human> rat > monkey> mini-pig> rabbit.

b. The data from this study will be used to establish a physiologically-based pharmacokinetic (PBPK) model for the human. PBPK models are useful in predicting the internal dose of toxic moiety of chemicals. USEPA has advocated the application of *in vitro* biotransformation data and pharmacokinetic modeling to risk assessment. These data will be useful in providing experimental data regarding a component of the model that estimates RDX clearance rates and will result in a model that more accurately predicts safe levels of exposure for human exposed to environmental concentrations of RDX.

c. The USACHPPM, DTOX, Health Effects Research Program (HERP) is involved in the

development of novel *in vitro* methods for early testing and fast screening of proposed new energetic compounds. The work described here established an *in vitro* human metabolic model, which mimics the *in vivo* physiological condition and that is useful in the evaluation of metabolic fate for novel energetics, such as formulations to replace RDX and for other toxic industrial chemicals/toxic industrial materials (TICs/TIMs).

3. RECOMMENDATIONS: This *in vitro* metabolic model provided data important in understanding the transport and clearance of assimilated oral RDX exposures for human risk assessment. It is recommended that these methods be used in conjunction with verified animal PBPK and toxicity information in refining risk-based screening levels and in the further identification of metabolites and metabolic pathways.

Toxicology Study No. 87-XE-0982-09; *In Vitro* Study of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) Metabolism in Human Liver, Oct 2008

CONTENTS

List of Figures

List of Tables

TOXICOLOGY STUDY NO. 87-XE-0982-09
IN VITRO STUDY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) METABOLISM IN HUMAN LIVER

1. REFERENCES. See Appendix A for a listing of references.

2. AUTHORITY. To ensure environmental safety and occupational health (ESOH) as part of the responsibilities outlined in Army Regulation (AR) 200-1 (reference 1), and occupational health through AR 40-5 (reference 2) and AR 70-1 (reference 3), this study, sponsored by U.S. Army Corps of Engineers Engineering Research and Development Center (ERDC), was completed as a work unit of pharmokinetics and toxicodynamics of RDX within the Toxicogenomics for Assessment of Munitions Constituents Program.

3. PURPOSE. This project addresses RDX metabolism in human liver. These studies were based on the use of human tissues and cells to investigate RDX metabolism in human liver in support of the proposed USEPA re-assessment of the oral reference dose for RDX. The developed *in vitro* human metabolic model, which mimics *in vivo* physiological condition, is useful in the evaluation of human metabolic fate for novel energetics such as formulations to replace RDX and for other toxic industrial compounds/toxic industrial materials (TICs/TIMs). These data will serve to reduce the uncertainty (factor of 10) from extrapolating rodent toxicity information to humans. This project provided *in vitro* human data in support of the work of pharmokinetics and toxicodynamics of RDX within the Toxicogenomics for Assessment of Munitions Constituents Program of the USAERDC.

4. GENERAL BACKGROUND.

a. The USEPA has established an oral reference dose (RfD) of 3 μg/kg/day for humans exposed to RDX, based on a study carried out in rats (Levine *et al*, 1983). Currently, the Directorate of Toxicology is reassessing the work on which the RfD is based, as part of a suite of studies that will be submitted to the USEPA in support of a refinement of the RfD. An important part of these studies is to provide data that reduce the uncertainty associated with extrapolating animal toxicity information to humans. Traditionally, an arbitrary uncertainty factor of 10 is used; however, the USEPA supports the reduction of this value to 3 when PBPK models are employed. There are data relevant in understanding environmental breakdown and/or metabolism of RDX; however, only a few studies have reported RDX metabolism in animal species (e.g. mouse, rat and mini-pig). This project is to provide RDX clearance rates and metabolic profiles using human liver tissues, cells and recombinant human CYP450 isoforms to investigate whether and how RDX is metabolized by human liver and also compare the metabolic profiles between species, human, pig, monkey, rat and rabbit. These data will be used specifically to refine human PBPK models to estimate internal dose of RDX at the site of action.

b. Liver microsomes are subcellular fractions that contain drug-metabolizing enzymes including the cytochrome P450 (CYP) enzymes, flavin monooxygenases, and UDP glucuronyl transferases. Liver microsomes are a major tool for studying xenobiotic metabolism and drug-drug interactions. Human liver microsomes represent a well-accepted *in vitro* experimental system for the evaluation of human metabolic fate of xenobiotics. This project is the first to use human liver microsomes to measure the RDX metabolism to generate a human RDX metabolic profile useful and meaningful in the extrapolation of data useful in the assessment of human risk from RDX exposure. Cytochrome P450 (CYP450) is a key drug metabolizing enzyme and consists of a number of isoforms or isoenzymes. RDX has been reported to be metabolized by a CYP450 2B4 from rabbit liver. Humans do not have 2B4 but do have an ortholog, 2B6, which is generally believed to be comparable in many ways to 2B4. This study is to investigate which type of the CYP450 isoforms will be involved in RDX liver metabolism by testing various recombinant human CYP450 isoforms including the 2B6.

5. MATERIALS.

a. Test Article

Hexahydro-1,3,5-Trinitro-1,3,5-Triazine, RDX, was produced at Holston Army Ammunition Plant and analyzed by HPLC and determined to be >99.5% pure. A stock solution was prepared at least 100x in DMSO or acetonetrile to give a final concentration of the solvent at ≤ 1%. Medium controls contained the same amount of the solvent used in the samples.

b. Metabolites Reference

SRI International Chemical Sciences and Technology Department provided the following metabolites as standards:

- 4-nitro-2,4-diazabutanal (NDAB)
- 1-nitroso-3,5-dinitro-1,3,5-triazaine (MNX)
- 1,3-dinitroso-5-nitro-1,3,5-triazaine (DNX)
- 1,3,5-trinitroso-1,3,5-triazine (TNX)
- methylenedinitramine (MEDINA)

c. Liver Tissues and Cells

Human and animal liver microsomes, S9, human hepatocytes and recombinant human CYP450 isoforms were purchased from Invitrogen or BD Biosciences.

6. METHODS

a. Experimental Approach

(1) To test the initial hypothesis, that RDX is metabolized by human liver we screened RDX metabolism using the following tiered approach:

- human liver microsomes (LM)
- human liver S9
- human hepatocytes
- recombinant human cytochrome P450 (CYP) isoforms

(2) To prove RDX *in vitro* metabolism we designed and carried out studies using the strategies described below:

- The RDX tested in these studies covered a wide range from 50 μM to 800 μM to observe the dose-dependent response.
- Reactions were conducted at 37°C and terminated at 0, 30, 60, 120 or 240 minutes to observe the time-dependent response.
- Parent-loss tests and possible formation of metabolites was evaluated by HPLC, LC/MS/MS or GC/ECD using five RDX metabolite standards.

(3) To ensure the accuracy of this *in vitro* test system we performed:

- Positive and negative controls carried out in parallel.
- The initial study, a key study, was completed in compliance with Good Laboratory Practice (GLP) Standards.

(4) To compare the effects of RDX on CYP450 activity another traditional explosive compound, 2,4,6-Trinitrotoluene (TNT), was tested in these experiments.

b. Procedures

(1) In general, RDX was incubated with human and animal LM, S9, hepatocytes or recombinant CYP450 isoforms liver microsomes at 37°C at different concentrations: 50 - 800 μM, for different incubation times: 0, 30, 60, 120 and 180 minutes in the absence and presence of NADPH or by using activated and inactivated tissues. Incubation was also performed without microsomes to evaluate chemical stability. The reactions were conducted under aerobic, anaerobic and oxygen-reduced conditions to understand the mechanism of RDX metabolism.

(2) The parent test article concentrations at the various initial concentrations and incubation times were quantified by HPLC, GC/ECD and LC/MS/MS. The possible formation of metabolites was evaluated using five metabolite standards.

(3) CYP450 activity of LM was determined by EROD (Ethoxyresorufin O–Deethylase) assay using either ethoxyresorufin or benzyresorufin as the substrate.

(4) Specific activity of a recombinant human CYP450 1A2 was determined by measuring luminescence using P450-Glo™ CYP1A2 Screening system (Promega #V9770).

7. RESULTS.

a. Quantitative Control of RDX Dosing Concentrations

The initial concentrations of RDX applied to liver microsomes and S9 were at 40, 200 and 1000 µM. Following metabolic reactions, supernatants were collected by microcentrifuge and determined for the dosing concentrations of RDX by HPLC. The results show that both concentrations were very close at zero time indicating 1) the accuracy of preparation of the dosing concentrations of RDX and performance of the dosing and 2) the suitable practicability of the procedures/methods used for extraction of RDX from the RDX-dosed tissues and chemical analysis (Table 1).

Table 1. Quantification of RDX dosing concentrations

Samples (0 time)	**RDX dosing (µg/mL) in microsomes and S9**		**RDX (ppm) in microsomes by HPLC**		**RDX (ppm) in S9 by HPLC**	
	individual	**average**	**individual**	**average**	**individual**	**average**
0-1	8.88	8.88 (40 µM)	8.417	9.36	8.925	9.16
0-2	8.88		10.301		9.387	
0-3	44.4	44.40 (200 µM)	39.55	38.15	46.31	45.92
0-4	44.4		36.68		45.532	
0-5	222.1	222.10 (1000 µM)	208.7	221.3	237.118	229.88
0-6	222.1		233.9		222.64	

b. RDX Metabolism Screening in Various Tissues and Different Conditions

These studies were conducted in different laboratories with in-house and outside collaborations. An initial key study was completed in compliance with GLP Standards. Table 1 summarized the major significant findings collected from these collaborators. Neither parent RDX loss nor its metabolites were detected in human liver microsomes, S9, hepatocytes and a number of human recombinant CYP450 isoforms under aerobic condition. Further study was carried out under anaerobic condition with nitrogen replacing oxygen. It was found that RDX was metabolized in a number of human recombinant CYP450 isoforms and one of the RDX metabolites, MEDINA, was detected under such anaerobic conditions. This result is comparable to RDX's significant anaerobic metabolism in various microorganisms.

Table 2. Summary of RDX *in vitro* metabolism under aerobic and anaerobic conditions

Human tissues		RDX parent loss		RDX metabolites	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Microsomes	sex mix pooled	–	NT*	–	NT*
	male pooled	–	NT*	–	NT*
	female pooled	–	NT*	–	NT*
S9	sex mix pooled	–	NT*	–	NT*
Hepatocytes	sex mix pooled	–	NT*	–	NT*
Recombinant CYP450 Isoforms	1A1	–	+	–	–
	2B6	–	+	–	+ MEDINA
	2C8	–	+	–	+ MEDINA
	2C18	+	+	–	+ MEDINA
	2E1	+	+	–	+ MEDINA

	3A5	-	+	–	+ MEDINA
	Mix	–	+	–	+ MEDINA

*: Not Tested

c. CYP450 Activity in Human and Animal Liver Microsomes and Effects of RDX

These studies measured the basal CYP450 activity in human and various animal liver microsomes and the effect of RDX on CYP450 activity. Interestingly, the monkey LM had significantly higher CYP450 activity than others, especially than humans (Figure 1). RDX showed no inhibition, unlike TNT, on CYP450 activity of human and animal liver microsomes (Figure 2) and human recombinant CYP450 1A2 (Figure 3). This testing encouraged us to stay with our initial hypothesis and develop/improve specific experimental conditions to determine RDX *in vitro* metabolism in human tissues.

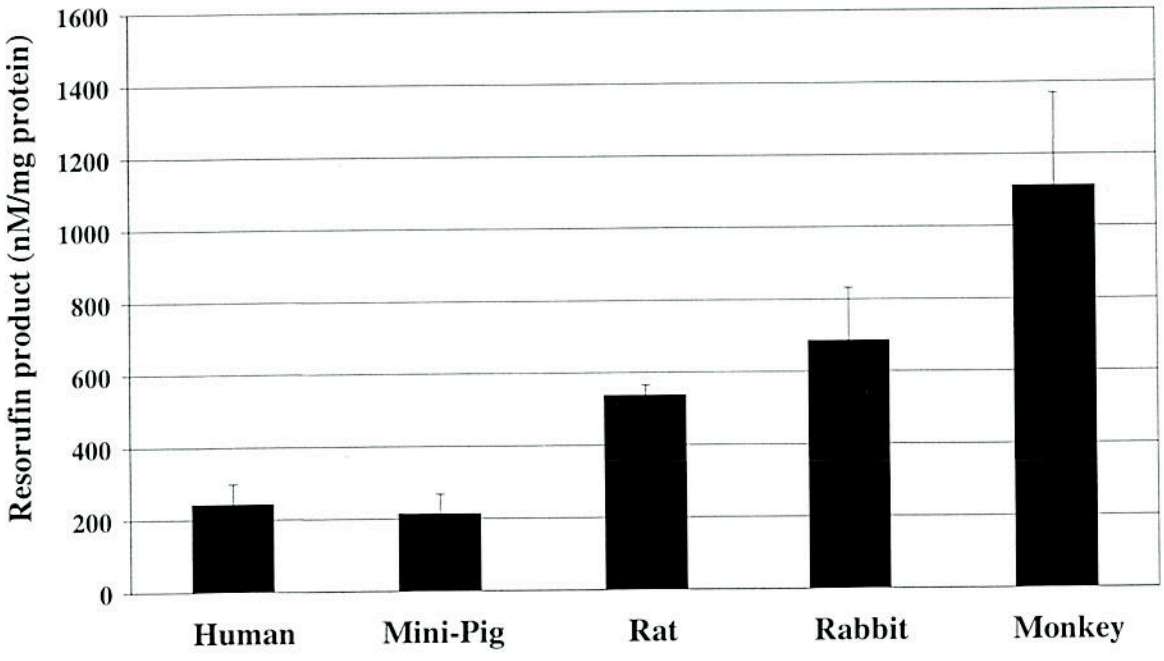

Figure 1. CYP450 activity of human and animal liver microsomes by EROD assay

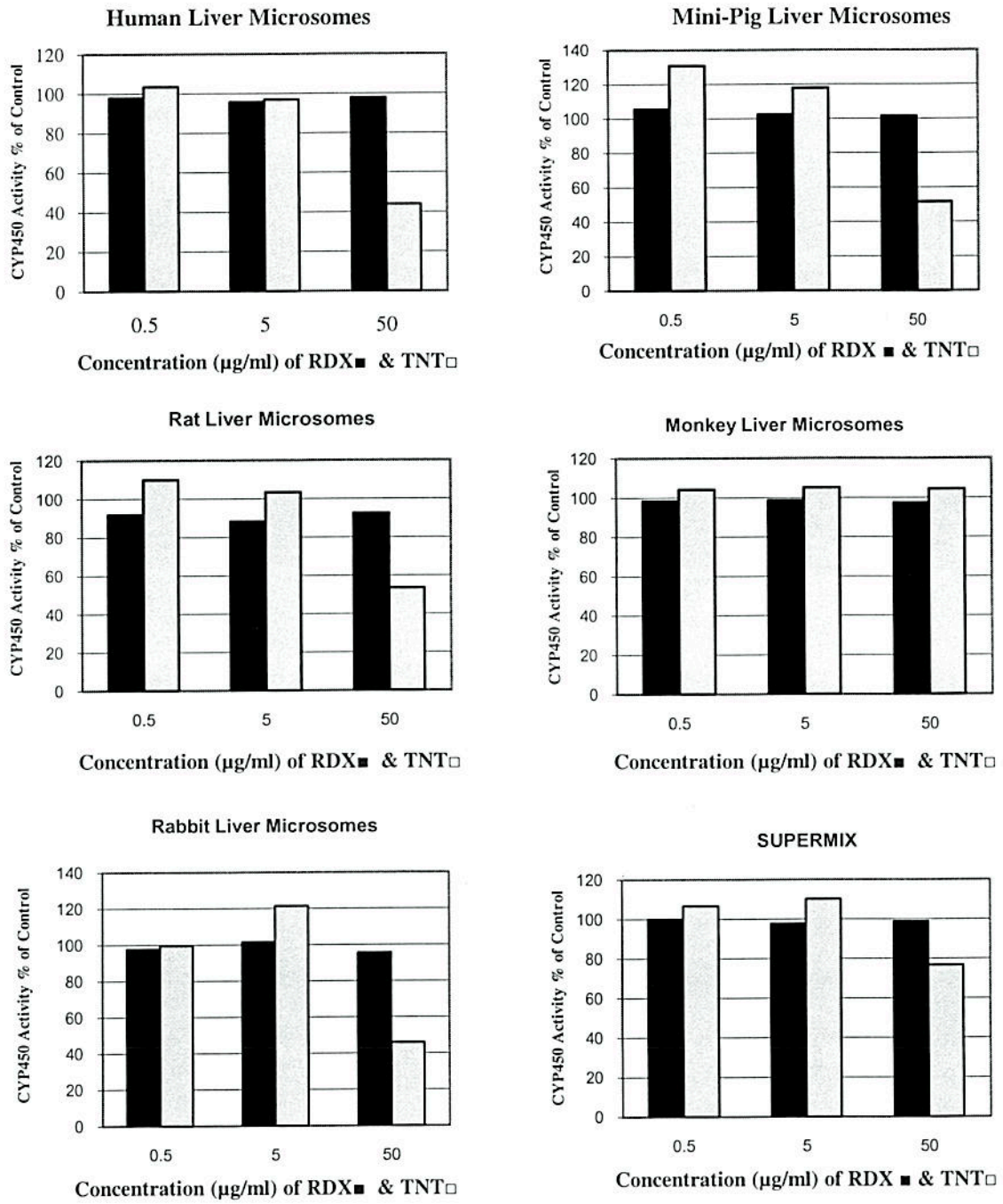

Figure 2. Mean CYP450 activity on human and animal liver microsomes as a function of RDX (■) and TNT (□) concentrations, respectively

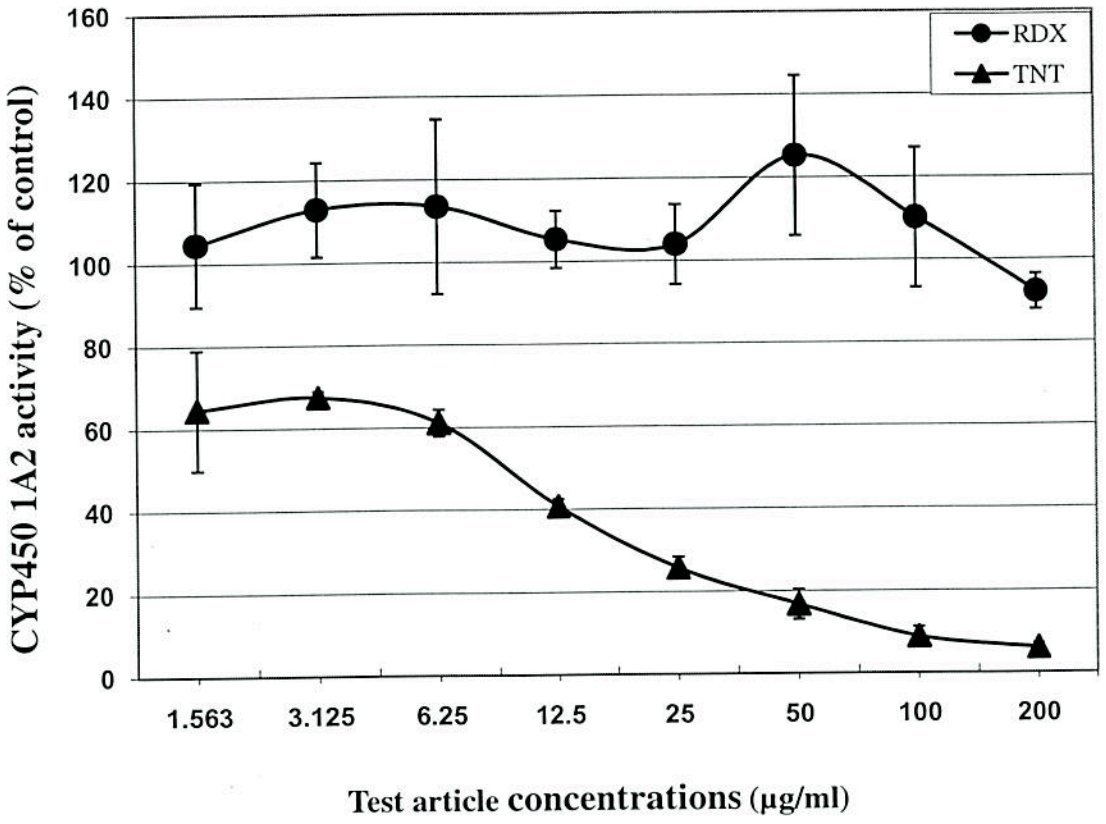

Figure 3. Mean activity on recombinant human CYP450 1A2 isoform as a function of RDX and TNT concentrations, respectively. I-bars represent the data collected from two individual experiments.

d. RDX Metabolism in Human and Animal Liver Microsomes (LM) under Oxygen-Reduced Condition

The reaction of RDX with human and several animal LM was carried out in an oxygen-reduced incubation and terminated at various times. The amount of RDX remaining in the reaction mixtures was measured as a function of incubation times. Under the improved condition, RDX was significantly metabolized in all tested species (Figures 4 and 5 and Table 3). Loss of the parent compound (RDX) was determined at 30 and 180 minutes of the incubation and ranked as human (46.6% & 51.8%)> rat (40.1% & 47.2%)> monkey (34.6% & 35.7%)> mini-pig (25.5% & 33.7%)> rabbit (11.6% & 18.0%). Metabolic rate of RDX showed the same ranking as

the percentage of the parent compound loss in all liver microsomes from the human and animals (Table 3).

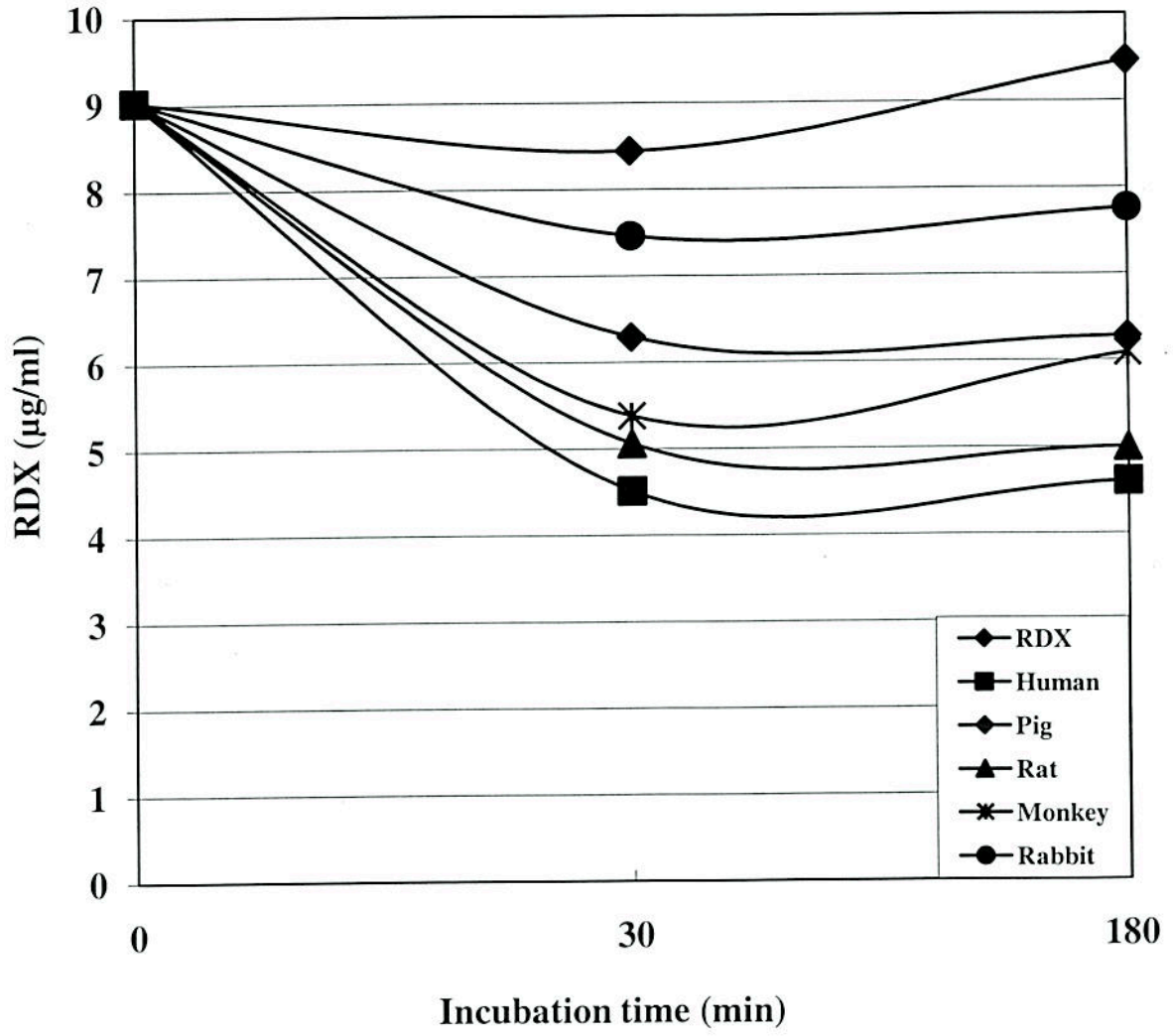

Figure 4. Mean RDX concentrations in human and animal liver microsomes determined at 0, 30 and 180 min incubation under oxygen-reduced condition

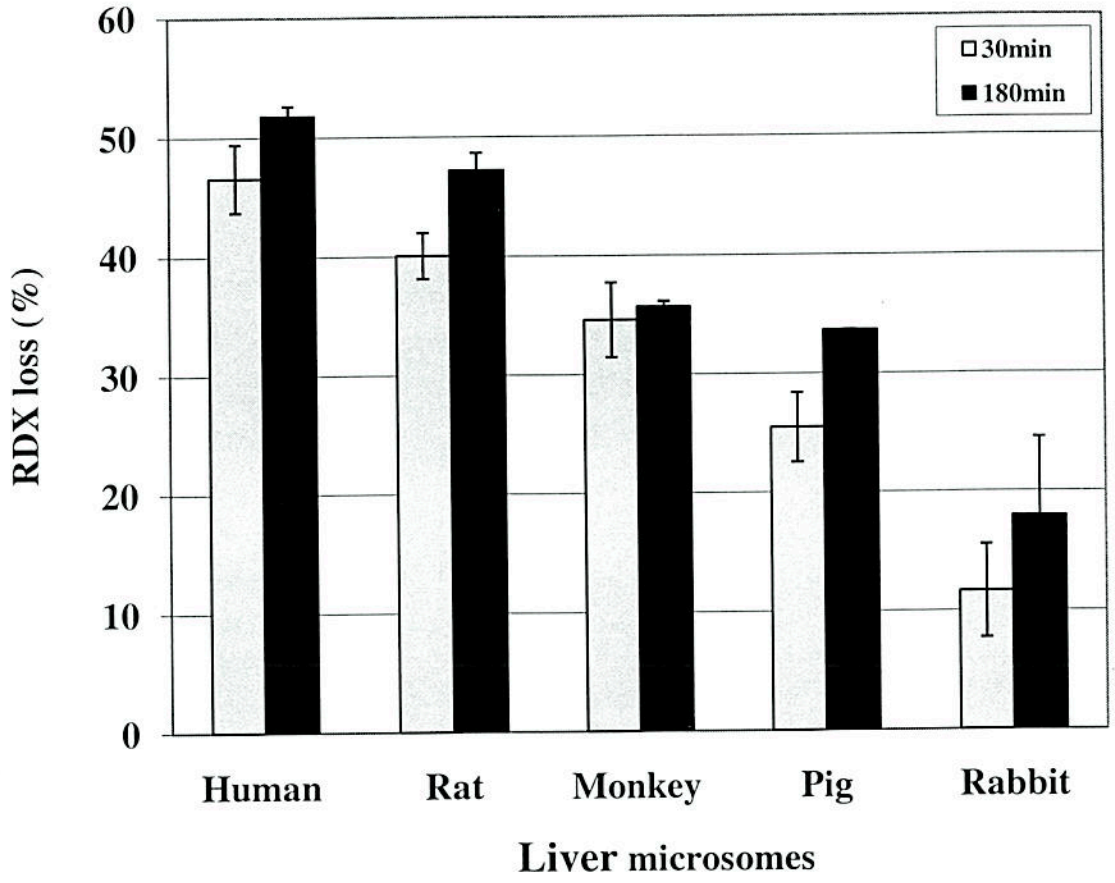

Figure 5. RDX parent loss (%) in human and animal liver microsomes at 30 and 180 min incubation, respectively

Table 3. RDX metabolic rate in human and animal liver microsomes

Microsomes (LM)	30 min Metabolized RDX (ng RDX/mg protein/min)		180 min Metabolized RDX (ng RDX/mg protein/min)	
	Mean	SD	Mean	SD
Human LM	131.033	8.026	26.648	0.635
Rat LM	112.747	5.428	24.236	1.133
Monkey LM	105.242	6.663	18.767	0.241
Mini-Pig LM	79.542	8.954	17.701	0.009
Rabbit LM	32.738	11.007	9.452	3.453

8. DISCUSSION

a. These studies were conducted with in-house and outside collaborations. An initial key study was completed in compliance with Good Laboratory Practice (GLP) guidelines. Tables 1 and 2 summarized the major significant findings collected from these collaborators. Neither parent RDX loss nor its metabolites were detected in human liver microsomes, S9, hepatocytes and a number of human recombinant CYP450 isoforms under aerobic condition.

b. RDX showed no inhibition, unlike TNT, on CYP450 activity of human and animal liver microsomes (Figure 2) and human recombinant CYP450 1A2 (Figure 3). These results are consistent with our initial hypothesis to develop/improve specific experimental conditions that are more consistent with human tissue-specific metabolism conditions.

c. Further work was carried out under anaerobic conditions with nitrogen replacing oxygen. It was found that RDX was metabolized in a number of human recombinant CYP450 isoforms and one of the RDX metabolites, MEDINA, was detected under such anaerobic conditions (Table 2). This result is comparable to the remarkable anaerobic metabolism of RDX in various microorganisms.

d. Oxygen concentrations in acinar zone 1 of the liver (first entry of blood) are 9-13%, but only 4-5% in zone 3, where the predominant P450 enzyme concentrations are found. Since zone 3 is relatively hypoxic, rates and products of metabolism may be affected. To mimic such *in vivo* physiological condition we reduced the amount of oxygen in the incubator when RDX was incubated with human and animal microsomes and found that RDX was significantly metabolized in all tested species (Figures 4 and 5 and Table 3). Loss of the parent compound (RDX) was determined at 30 and 180 minutes of the incubation and ranked as human (46.6% & 51.8%)> rat (40.1% & 47.2%)> monkey (34.6% & 35.7%)> Pig (25.5% & 33.7%)> rabbit (11.6% & 18.0%). The rate of metabolism of RDX (Table 3) maintained the same order of ranking as the percentage of the parent compound lost in liver microsomes from the human and animals (Figure 5). Further characterization of profiles of the RDX *in vitro* metabolism, such as identifications of the metabolites and pathways, e.g., which CYP450 isoform(s) play a key role in RDX metabolism, are underway.

e. The data from this study will be used to provide the rate of metabolism, a key input for the human PBPK model. The PBPK models are useful in predicting the internal dose of toxic moiety of chemicals. USEPA has advocated the application of *in vitro* biotransformation data and pharmacokinetic modeling to risk assessment. These data will be used in this extrapolation effort to provide a more accurate characterization of systemic RDX concentration at the site of toxic effect (i.e. the brain) following oral exposures.

f. This project provided marked improvement of the methods currently used in conventional *in vitro* metabolic assays through an understanding and development of a liver physiological condition (low oxygen) consistent with those in an *in vivo* system. This model will be useful in the evaluation of human metabolic fate for novel energetics and for a wide range of other TICs/TIMs.

9. CONCLUSION

a. This effort involved estimating metabolic rate of RDX disappearance *in vitro* using human tissue including S9 preparations, microsomes, hepatocytes and several recombinant CYP450 isoforms under aerobic, anaerobic and oxygen reduced conditions. The RDX metabolism was also conducted in several animal liver microsomes to compare with human liver microsomes. Metabolism of RDX was determined after 30 and 180 minutes of the incubations in these enzymatic systems with disappearance rates ranked from greatest to lowest as human> rat > monkey> mini-pig> rabbit.

b. The data from this study will be used in physiologically-based pharmacokinetic (PBPK) models for human risk assessment predictions. The PBPK models are useful in predicting the internal dose of toxic moiety of chemicals at the toxic site of action. The USEPA

has advocated the application of *in vitro* biotransformation data and pharmacokinetic modeling in risk assessment.

c. The work described herein established an *in vitro* human metabolic model, which mimics the *in vivo* physiological condition of the *in vivo* liver and that is useful in the evaluation of metabolic fate for other novel energetics, such as replacement formulations for RDX and for other toxic industrial chemicals/toxic industrial materials (TICs/TIMs).

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APPENDIX A

1. References Cited:

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b. Army Regulation 40-5, Preventive Medicine, 25 may 2007.

c. Army Regulation 70-1, Army Acquisition Policy, 31 December 2003.

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